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Increase in the Phosphotransferase Specific Activity of Purified Rous Sarcoma Virus pp60$^{v\text{-}src}$ Protein After Incubation with ATP Plus Mg^{2+}

A. F. PURCHIO, SUSAN K. WELLS, AND MARC S. COLLETT*

Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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pp60$^{v\text{-}src}$, the product of the Rous sarcoma virus *src* gene, was partially purified by immunoaffinity chromatography from extracts of Rous sarcoma virus-transformed field vole cells. Incubation of this preparation with ATP plus Mg^{2+} and subsequent repurification by chromatography on hexylamine-agarose resulted in a net increase in the specific activity of the *src* protein kinase. This increase in phosphotransferase activity was detected by using a variety of substrates including casein, tubulin, and a 34,000-dalton protein presumed to be an in vivo target substrate of pp60$^{v\text{-}src}$. In all cases, the phosphorylation was at tyrosine residues, and the kinase activity was inhibited by preincubation of the enzyme with immunoglobulin G prepared from tumor-bearing rabbit sera. The implications of these results for the regulation and control of pp60$^{v\text{-}src}$-associated kinase activity are discussed.

The product of the Rous sarcoma virus (RSV) *src* gene is a 60,000-dalton (60K) phosphoprotein termed pp60$^{v\text{-}src}$ (2, 22). Early experimental results suggested that a tentative function of this transforming protein was that of a protein kinase. This assignment was based on the ability of immune complexes containing pp60$^{v\text{-}src}$ to phosphorylate the heavy chain of immunoglobulin G (IgG) molecules directed against the pp60$^{v\text{-}src}$ protein (5, 16). Subsequent purification of pp60$^{v\text{-}src}$ from cytoplasmic extracts of RSV-transformed cells indicated that pp60$^{v\text{-}src}$ either was itself a protein kinase or was tightly associated with a protein kinase (9, 10). More recently, the *src* gene has been molecularly cloned to expression in *Escherichia coli* (11, 12, 18). The polypeptide produced in bacteria appears to possess protein phosphotransferase activity (11, 18), clearly indicating that the RSV-transforming protein pp60$^{v\text{-}src}$ is indeed a protein kinase. In all cases studied to date, the pp60$^{v\text{-}src}$ protein kinase activity appears to be specific for only tyrosine residues (6, 14, 17).

Early experiments with pp60$^{v\text{-}src}$ indicated that this enzyme was capable of apparent autophosphorylation (9); this was based on the observation that the addition of [γ-^{32}P]ATP to partially purified preparations of pp60$^{v\text{-}src}$ resulted in the phosphorylation of pp60$^{v\text{-}src}$ itself, although other workers have been unable to detect this activity (17). Experiments with highly purified preparations of pp60$^{v\text{-}src}$ suggest that pp60$^{v\text{-}src}$ either does phosphorylate itself or is very tightly associated with a second kinase which is responsible for phosphorylating pp60$^{v\text{-}src}$ (21). Analyses of pp60$^{v\text{-}src}$ phosphorylation in vitro indicate that the same major tyrosine residue is phosphorylated on pp60$^{v\text{-}src}$ in vivo (28).

Understanding the regulation of the pp60$^{v\text{-}src}$ kinase activity is an important step in understanding the mechanism of transformation by RSV. In this study, partially purified pp60$^{v\text{-}src}$ was incubated with ATP plus Mg^{2+} (ATP/Mg^{2+}) and, after separation from all unlabeled ATP, was analyzed for its kinase activity. We observed a significant increase in the tyrosine-specific protein kinase activity of the enzyme so treated on a variety of substrates compared with enzyme preincubated without ATP or with ATP plus Mg^{2+} plus EDTA (ATP/Mg^{2+}/EDTA).

MATERIALS AND METHODS

Cells and virus. The cells used in these experiments were European field vole (*Microtus agrestis*) cells transformed by the subgroup D Schmidt-Ruppin strain of RSV (clone 1-T, originally provided by A. J. Faras, University of Minnesota Medical School). Cells were grown in Dulbecco modified Eagle medium containing 5% calf serum. To radiolabel cell proteins, cells were incubated for 2 to 3 h in methionine-minus medium containing 100 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) per ml before harvesting.

Purification of pp60$^{v\text{-}src}$ and incubation with ATP/Mg^{2+}. pp60$^{v\text{-}src}$ was prepared from lysates of 1-T

cells by immunoaffinity chromatography exactly as described (9, 10), except that 1 M KSCN was used to elute the enzyme. A typical preparation of pp60$^{v\text{-}src}$ from 24 100-mm dishes of 1-T cells (approximately 5 × 10^8 cells) resulted in 0.9 ml of enzyme preparation after dialysis against 50% glycerol. These preparations (400 μl containing about 30 μg of protein) were incubated with 100 μM ATP (P-L Biochemicals, Inc., Milwaukee, Wis.)–10 mM $MgCl_2$–0.01 M Tris-hydrochloride (pH 7.2) in a total volume of 1.2 ml for 60 to 90 min at 22°C. Reactions were terminated by the addition of EDTA (adjusted to pH 7.2) to a final concentration of 10 mM and applied to a 1 -ml column of hexylamine-agarose (P-L Biochemicals) preequilibrated with buffer A (20 mM potassium phosphate [pH 7.2]–0.001 M EDTA–0.01% Nonidet P-40–10% glycerol) at 4°C. The column was washed with 40 ml of buffer A containing 250 mM KCl to remove all free ATP. We have found that this procedure is more efficient in the removal of ATP than extensive dialysis. pp60$^{v\text{-}src}$ was then eluted with buffer A plus 1 M KCl and immediately dialyzed against 20 mM potassium phosphate (pH 7.2)–1 mM EDTA–50% glycerol for 20 h at 4°C. The recovery of pp60$^{v\text{-}src}$ protein was routinely 25%. We presume that these losses were due to nonspecific adsorption to the column or dialysis bag as a consequence of the low concentration of protein being handled. Enzymes were stored at −20°C and were found to be stable for over 6 months.

For control reactions, pp60$^{v\text{-}src}$ was incubated either with Mg^{2+} alone or with ATP/Mg^{2+}/EDTA (10 mM, pH 7.2) and processed exactly as described above. Since pp60$^{v\text{-}src}$ was labeled with [^{35}S]methionine, we were able to assure ourselves that we were recovering equal amounts of the pp60$^{v\text{-}src}$ protein after hexylamine-agarose chromatography of the various control and experimental reactions by electrophoresing a sample of the enzyme preparation on a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel (15), localizing the pp60$^{v\text{-}src}$ band by fluorography (3), and cutting out and counting the band by liquid scintillation spectrometry.

Determination of the amount of pp60$^{v\text{-}src}$ protein present in protein kinase reactions. To estimate the molar quantity of pp60$^{v\text{-}src}$ polypeptide present in our protein kinase reactions, cells were radiolabeled with [^{35}S]methionine (50 μCi/ml) over a period of 48 h with one change of fresh radiolabel-containing medium. This was done to approach a steady-state labeling of cellular proteins. pp60$^{v\text{-}src}$ was then purified from these cells by our standard immunoaffinity chromatography procedure. A known volume of this [^{35}S]methionine-labeled enzyme preparation was electrophoresed in an SDS-polyacrylamide gel. The pp60$^{v\text{-}src}$ protein band was localized by fluorography and excised, and the amount of radioactivity present was quantified by liquid scintillation spectrometry. Knowing the specific activity of the total [^{35}S]methionine-labeled cellular protein allowed us to estimate that approximately 0.47 pmol of pp60$^{v\text{-}src}$ polypeptide from our immunoaffinity-purified enzyme preparations was present in our standard protein kinase reactions. This approach to the estimation of the molar amount of pp60$^{v\text{-}src}$ present in our reactions may be slightly in error to the extent that the specific methionine radioactivity of steady-state labeled total cellular protein differs from that of pp60$^{v\text{-}src}$. The amount of pp60$^{v\text{-}src}$ protein present in analytic reactions comparing the A enzyme with the B enzyme (see Fig. 4 and 6) was approximately 25% of the above value.

Kinase reactions. Standard analytic protein kinase reactions were carried out in a total volume of 30 μl containing 10 mM $MgCl_2$, 0.01 M Tris-hydrochloride (pH 7.2), and 1.0 μM [γ-^{32}P]ATP (600 Ci/mmol), an appropriate amount of substrate as indicated in the figure legends, and 5 μl of pp60$^{v\text{-}src}$ enzyme preparation. Incubations were for 30 min at 22°C unless otherwise indicated, and reactions were terminated by the addition of 30 μl of 2× SDS-sample buffer and boiled for 1 min. Samples were fractionated on 10% polyacrylamide-SDS gels (15), which were then stained, destained, dried, and autoradiographed with Cronex X-ray film and Lightning-Plus intensifying screens (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.). For quantitation, appropriate bands were cut from the dried gels and counted by liquid scintillation spectrometry.

Partial purification of a 34,000-dalton cellular protein substrate of pp60$^{v\text{-}src}$. The purification of the putative 34,000-dalton (34 K) cellular protein substrate of pp60$^{v\text{-}src}$ followed a previously described procedure (8) with the following modifications. After application of the DEAE flow-through material to a column of hydroxylapatite, this column was washed with column buffer (10 mM Tris-hydrochloride [pH 7.2]–1 mM EDTA–1 mM 2-mercaptoethanol–0.05% Nonidet P-40–10% glycerol) containing 0.2 M potassium phosphate. The 34K protein was then eluted with column buffer containing 0.5 M potassium phosphate. This eluted material was dialyzed for 16 h at 4°C against column buffer containing 50% glycerol, diluted fourfold, and then applied to a second DEAE-Sephacel column. The 34K protein-containing fractions in the flow-through were pooled and used as substrate for the pp60$^{v\text{-}src}$ kinase.

RESULTS

Figure 1, track 1, shows an SDS-polyacrylamide gel analysis of pp60$^{v\text{-}src}$ purified from [^{35}S]methionine-labeled 1-T cells by immunoaffinity chromatography. Although essentially only one major band can be seen with fluorography, silver staining (19) of these gels reveals the presence of several other protein bands which presumably contain little or no methionine or are poorly radiolabeled under these conditions (21). This in vivo radiolabeling with [^{35}S]methionine allows us to monitor the recoveries of pp60$^{v\text{-}src}$ in subsequent experiments. Incubation of pp60$^{v\text{-}src}$ prepared in this fashion with [γ-^{32}P]ATP resulted in the phosphorylation of pp60$^{v\text{-}src}$(Fig. 1, track 2,) and this labeling could be prevented by the addition of excess unlabeled ATP (data not shown). Also shown is the phosphorylation of casein by pp60$^{v\text{-}src}$ protein kinase (Fig. 1, track 3).

The protein kinase reactions shown in Fig. 1, tracks 2 and 3, were performed at a [γ-^{32}P]ATP concentration of 1 μM. This concentration and even lower concentrations have been routinely used in the study of pp60$^{v\text{-}src}$ phosphorylation.

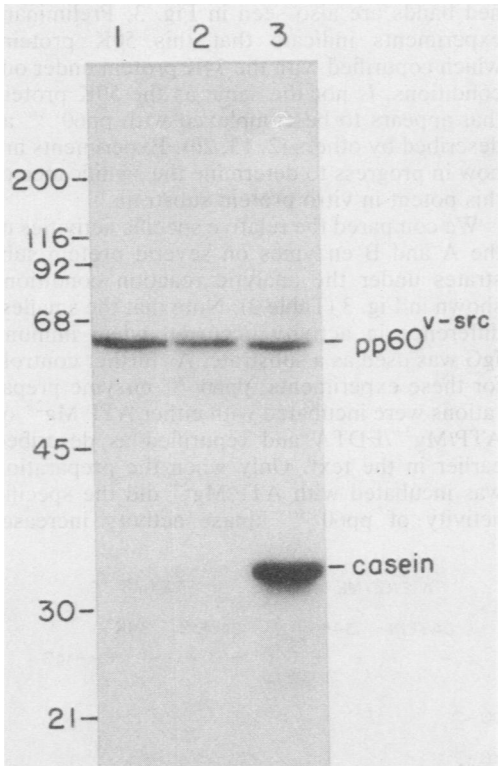

FIG. 1. Polyacrylamide gel analysis of immunoaffinity-purified pp60$^{v\text{-}src}$. [^{35}S]methionine-labeled 1-T cell lysates were subjected to immunoaffinity chromatography on a column containing tumor-bearing rabbit serum IgG, as described in the text. Track 1 is a fluorogram of a gel of the [^{35}S]methionine proteins (approximately 10,000 cpm) present in the pooled, dialyzed KSCN eluate fractions. Tracks 2 and 3 are autoradiographic representations of pp60$^{v\text{-}src}$ protein kinase activity resolved on a second polyacrylamide gel. Track 2 contains the enzyme preparation incubated with 1 μM [γ-^{32}P]ATP/Mg^{2+} alone; track 3 contains 1 μM [γ-^{32}P]ATP/Mg^{2+} plus 1 mg of casein per ml in standard analytic kinase reactions as described in the text. The amount of ^{35}S-labeled pp60$^{v\text{-}src}$ enzyme present in the protein kinase reactions (<100 cpm) was insufficient to be detected by the autoradiographic exposure for detection of ^{32}P-labeled kinase reaction products.

However, this value is considerably below the previously estimated K_m value of the enzyme for ATP (approximately 20 μM; 6, 26). Since we were interested in studying the possible effect on pp60$^{v\text{-}src}$ enzyme activity of incubation with ATP, we have compared the phosphorylation (autophosphorylation) of pp60$^{v\text{-}src}$ at this normally used low ATP concentration with kinase reactions performed at a much higher ATP concentration. Table 1 shows the molar ratio of phosphate incorporation into the pp60$^{v\text{-}src}$ polypeptide at two different concentrations of ATP, 1 and 100 μM. From estimating the molar amount of pp60$^{v\text{-}src}$ polypeptide in our enzyme preparations (see above) and the amount of radioactivity present in the phosphorylated pp60$^{v\text{-}src}$ protein band, we found that there was approximately a sixfold increase in the phosphorylation of pp60$^{v\text{-}src}$ when reactions were conducted at 100 μM ATP compared with those at 1 μM ATP. These data indicate that a considerable proportion, if not all, of the pp60$^{v\text{-}src}$ molecules in reactions performed at 100 μM ATP were phosphorylated. Figure 2 shows the time course for the in vitro phosphorylation reaction of pp60$^{v\text{-}src}$ conducted at 100 μM ATP. Approximately 90% of the labeling was completed by 60 min of incubation, although this time did vary slightly from preparation to preparation. The increased labeling of pp60$^{v\text{-}src}$ with incubation time (shown in Fig. 2) was due to increased phosphorylation and did not involve a phosphate exchange reaction. We have attempted to demonstrate such a potential exchange reaction with ^{32}P-labeled pp60$^{v\text{-}src}$; under all conditions tested, we have been unable to show a loss of radiolabel from pp60$^{v\text{-}src}$ (unpublished data).

Since pp60$^{v\text{-}src}$ was capable of incorporating considerable amounts of phosphate at the higher ATP concentration, we examined the effect of preincubation of pp60$^{v\text{-}src}$ with 100 μM ATP on the kinase activity of the enzyme. Equal amounts of partially purified pp60$^{v\text{-}src}$ protein were incubated with Mg^{2+} and unlabeled ATP at 100 μM (B enzyme) or with Mg^{2+} alone (A

TABLE 1. Extent of autophosphorylation of pp60$^{v\text{-}src}$ at two ATP concns[a]

ATP concn (μM)	mol of PO_4/mol of p60
1	0.17 ± 0.03
100	1.12 ± 0.29

[a] Protein kinase reactions, as described in the text, contained 0.47 pmol of immunoaffinity-purified pp60$^{v\text{-}src}$ polypeptide and [γ-^{32}P]ATP at final concentrations of either 1 μM (600 Ci/mmol) or 100 μM (21 Ci/mmol). After a 2-h incubation period, reactions were terminated, and samples were subjected to polyacrylamide gel electrophoresis. Phosphorylated pp60$^{v\text{-}src}$ was localized by autoradiography and excised, and the radioactivity was quantified by scintillation spectrometry. The molar amount of phosphate incorporated into the pp60$^{v\text{-}src}$ polypeptide was determined from the specific activity of the [γ-^{32}P]ATP. The amount of pp60$^{v\text{-}src}$ polypeptide present in the reactions was estimated by calculating the specific activity of steady-state [^{35}S]methionine-radiolabeled total cellular protein and the amount of [^{35}S]methionine radioactivity in the pp60$^{v\text{-}src}$ polypeptide band (as resolved by polyacrylamide gel electrophoresis) of a known volume of enzyme preparation. Values represent the average of several determinations.

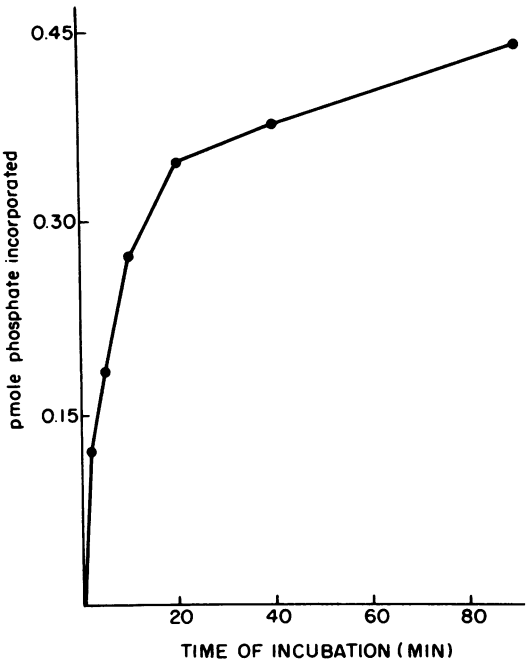

FIG. 2. Time course of the incorporation of radiolabel into pp60$^{v\text{-}src}$ incubated with [γ^{32}P]ATP/Mg^{2+}. Partially purified pp60$^{v\text{-}src}$ protein kinase (described in Fig. 1) was incubated at 22°C in the presence of 100 μM [γ-^{32}P]ATP plus 10 mM $MgCl_2$. At various times, equal portions of the reaction mixture were removed (equivalent to approximately 0.47 pmol of p60 protein) to an equal volume of 2X SDS-sample buffer and then boiled for 1 min. These samples were then subjected to SDS-polyacrylamide gel electrophoresis. The p60 phosphorylated bands were localized by autoradiography of the dried gel and were cut and counted in a liquid scintillation counter.

enzyme). After 90 min of incubation, both samples were chromatographed in an identical fashion on hexylamine-agarose as described above. After extensive washing of the column, the enzymes were eluted with 1 M KCl, and the eluates were dialyzed against 50% glycerol buffer. Recoveries of both pp60$^{v\text{-}src}$ proteins were routinely identical (about 25%).

Figure 3 shows the results of comparing the protein kinase activity of the two enzyme preparations on two protein substrates, casein and 34K protein. As can be seen from the autoradiogram, a dramatic increase in kinase activity occurred as a result of the prior incubation of pp60$^{v\text{-}src}$ with ATP/Mg^{2+} (B enzyme) compared to the control enzyme incubated with Mg^{2+} (A enzyme). The activity of both A and B enzymes was completely inhibited by preincubation with immune IgG. A 50K protein band, which was heavily phosphorylated by pp60$^{v\text{-}src}$ in our 34K preparations, as well as other minor, unidentified bands are also seen in Fig. 3. Preliminary experiments indicate that this 50K protein, which copurified with the 34K protein under our conditions, is not the same as the 50K protein that appears to be complexed with pp60$^{v\text{-}src}$ as described by others (2, 13, 20). Experiments are now in progress to determine the significance of this potent in vitro protein substrate.

We compared the relative specific activities of the A and B enzymes on several protein substrates under the analytic reaction conditions shown in Fig. 3 (Table 2). Note that the smallest difference in activity occurred when immune IgG was used as a substrate. As further controls for these experiments, pp60$^{v\text{-}src}$ enzyme preparations were incubated with either ATP/Mg^{2+} or ATP/Mg^{2+}/EDTA and repurified as described earlier in the text. Only when the preparation was incubated with ATP/Mg^{2+} did the specific activity of pp60$^{v\text{-}src}$ kinase activity increase,

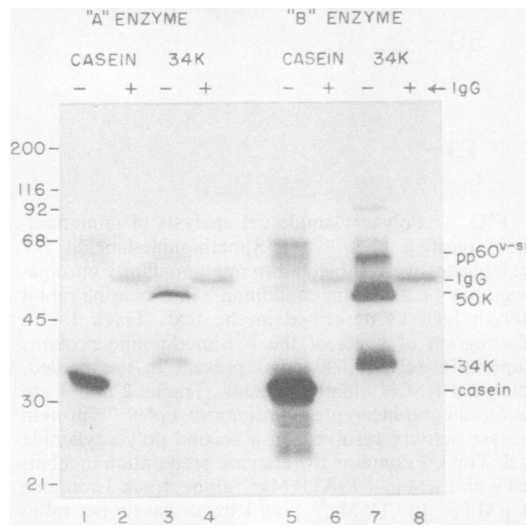

FIG. 3. Protein kinase activity of pp60$^{v\text{-}src}$ A and B enzyme preparations. Immunoaffinity-purified pp60$^{v\text{-}src}$ preparations were incubated in the presence of Mg^{2+} (A enzyme) or ATP/Mg^{2+} (B enzyme) and purified as described in the text. Equal amounts of pp60$^{v\text{-}src}$ in the resulting enzyme preparations were then used in standard analytic protein kinase reactions containing 1 μM [γ-^{32}P]ATP to phosphorylate either casein or 34K protein-containing preparations. Casein was present in reactions at 700 μg/ml, 34K was present at 70 μg/ml, and tumor-bearing rabbit serum IgG was present at 1 mg/ml. Tracks 1 through 4: A enzyme incubated with (1) casein, (2) casein plus IgG, (3) 34K, (4) 34K plus IgG. Tracks 5 through 8: B enzyme incubated with (5) casein, (6) casein plus IgG, (7) 34K, (8) 34K plus IgG. This figure represents an autoradiogram of a 10% polyacrylamide gel. No protein kinase activity was detected in reactions performed with either casein or 34K preparations incubated without the pp60$^{v\text{-}src}$ enzyme.

indicating that this observed effect depends on the addition of ATP/Mg^{2+} (data not shown).

Figure 4 shows that in all cases these phosphorylations took place exclusively on tyrosine residues. Furthermore, the phosphorylation sites on these protein substrates appeared to be similar whether the phosphorylation was carried out with the A or B enzyme. Figure 5 shows an autoradiogram of a two-dimensional fractionation of the tryptic phosphopeptides of the 34K protein phosphorylated by the A and B enzymes. The peptide maps are qualitatively very similar, indicating that the incubation of $pp60^{v\text{-}src}$ with ATP/Mg^{2+} does not radically alter the sites specificity of phosphorylation by the enzyme. However, there does appear to be a quantitative difference in the phosphorylation of one peptide; that is, one site (Fig. 5, arrow) is more highly phosphorylated by the A enzyme than by the B enzyme. The significance of this reproducible observation is not known at this time. It should further be noted that these tryptic phosphopeptide maps of the 34K protein are qualitatively similar to those previously published (23).

A time course for the phosphorylation of casein and the 34K protein is shown in Fig. 6A and B. Under the conditions of these analytic reactions (standard 1 μM ATP reactions), there was a five- and eightfold increase, respectively, in the activity of $pp60^{v\text{-}src}$ as a result of prior incubation with ATP/Mg^{2+} when casein and the 34K protein were used as phosphate acceptors. To more rigorously compare the kinetics of phosphorylation by the A and B enzyme preparations, however, we have determined the initial reaction velocities (V_0) for the phosphorylation of casein over a range of ATP concentrations. These V_0 values were then used to generate a Lineweaver-Burk plot of the reaction kinetics of the two enzymes (Fig. 6C). The linear kinetic profile of casein phosphorylation by the B enzyme appears to exhibit the standard Michaelis-Menten saturation of enzyme by ATP, revealing a K_m value for ATP of approximately 17 μM and a V_{max} value of 167 fmol · (min · pmol)$^{-1}$. The characteristics of casein phosphorylation by the A enzyme preparation, however, presented a different picture. The kinetics appeared to be nonlinear and in fact biphasic. At low ATP concentrations, the A enzyme exhibited high apparent V_{max} and K_m values [500 fmol · (min · pmol)$^{-1}$ and 150 μM, respectively]. As the ATP concentration increased, a break in the kinetic curve was observed in the range of 20 μM ATP, and the profile then more closely resembled that of the B enzyme. This change resulted in a substantial lowering of the apparent V_{max} value [92 fmol· (min · pmol)$^{-1}$)] and in a K_m value nearly identical to that of the B enzyme. These kinetic characteristics of the A

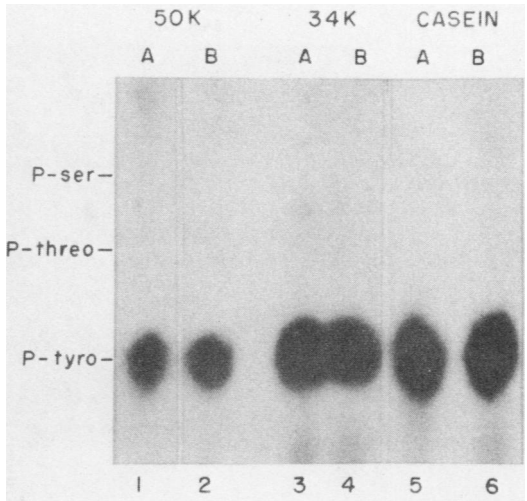

FIG. 4. Phosphoamino acid analyses of 50K and 34K proteins and casein phosphorylated by $pp60^{v\text{-}src}$ A and B enzymes. Various proteins phosphorylated as described in the legend to Fig. 3 were eluted from polyacrylamide gels and subjected to acid hydrolysis and paper electrophoresis at pH 3.5 as previously described (6). Authentic phosphoamino acid markers (ninhydrin stainable) were included in all samples. Tracks 1 and 2, 50K protein phosphorylated by (1) A enzyme and (2) B enzyme. Tracks 3 and 4, 34K protein phosphorylated by (3) A enzyme and (4) B enzyme. Tracks 5 and 6, casein phosphorylated by (5) A enzyme and (6) B enzyme. P-ser, Phosphoserine; P-threo, phosphothreonine; P-tyro, phosphotyrosine.

TABLE 2. Specific activity ratio of B enzyme to A enzyme with various phosphate acceptor proteins[a]

Protein substrate	Kinase sp act (B/A enzymes)
Casein	5.7 (3.7–7.8)
34K	11.5 (5.9–18.0)
50K	6.8 (3.8–8.2)
Tubulin	2.9 (2.2–3.6)
IgG	1.5 (1.0–2.0)

[a] $pp60^{v\text{-}src}$ A and B enzymes were prepared as described in the text. Each enzyme (containing identical amounts of p60 polypeptide) was incubated at 22°C for 30 min in standard analytic kinase reaction mixtures (as described in the text) containing various protein substrates. Reactions were terminated by the addition of SDS sample buffer, and samples were subjected to SDS-polyacrylamide gel electrophoresis. After autoradiographic localization of the various phosphorylated protein bands, the appropriate regions of the gel were excised, and the amount of radioactivity in the gel slices was determined by liquid scintillation counting. Values represent the average of several independent reactions employing three different preparations of the enzymes. Numbers in parentheses indicate the range of values obtained.

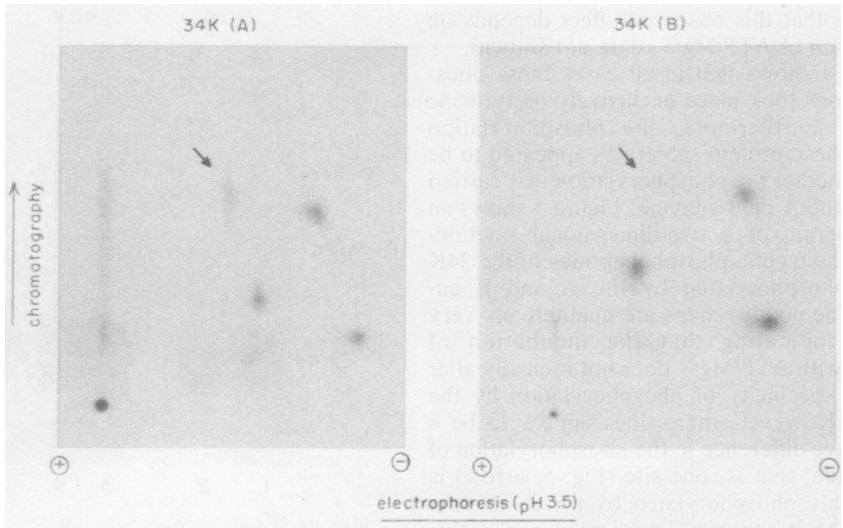

FIG. 5. Two-dimensional tryptic phosphopeptide fractionation of 34K protein phosphorylated by the pp60$^{v\text{-}src}$ A enzyme and B enzyme. The pp60$^{v\text{-}src}$ enzyme preparations were used to phosphorylate the 34K protein as described in the legend to Fig. 3. The 34K bands were excised from polyacrylamide gels, eluted, and processed for two-dimensional tryptic peptide mapping as previously described (4).

enzyme would be expected if during the course of the casein phosphorylation reaction the pp60$^{v\text{-}src}$ A enzyme were itself being converted to the B form by an ATP/Mg^{2+}-dependent mechanism.

DISCUSSION

Incubation of partially purified pp60$^{v\text{-}src}$ with ATP/Mg^{2+} and subsequent repurification on hexylamine-agarose resulted in a significant increase in the specific kinase activity of the enzyme as compared to that of controls incubated with ATP/Mg^{2+}/EDTA or with Mg^{2+} alone. We observed a 4- to 8-fold increase in the analytic phosphorylation of casein and as much as an 18-fold increase when the 34K protein, a putative in vivo target protein of pp60$^{v\text{-}src}$ (8, 24, 25), was used as substrate. In addition, a protein of 50,000 daltons which copurified with the 34K protein under our conditions served as an excellent substrate for pp60$^{v\text{-}src}$ kinase activity (Fig. 3). The significance of this new substrate will have to await in vivo studies to determine whether its phosphorylation state changes as a result of transformation with RSV. As shown in Fig. 4, all phosphorylations occurred at tyrosine residues, and all kinase activities were inhibited by IgG directed against pp60$^{v\text{-}src}$ (Fig. 3), indicating that the increased activity we observed was due to pp60$^{v\text{-}src}$.

These results have obvious implications with respect to the regulation of the pp60$^{v\text{-}src}$ kinase activity; therefore, it is important to determine exactly what is responsible for this increase in activity. The addition of [γ-^{32}P]ATP to pp60$^{v\text{-}src}$ results in the labeling of pp60$^{v\text{-}src}$ itself (Fig. 1), and this radiolabeling can be competed out by excess unlabeled ATP. Furthermore, this phosphorylation can be inhibited with antibody against pp60$^{v\text{-}src}$. Even with highly purified preparations of the enzyme, this in vitro phosphorylation occurs (21), suggesting that pp60$^{v\text{-}src}$ phosphorylates itself or is very tightly associated with a kinase which phosphorylates pp60$^{v\text{-}src}$.

We found that the extent of autophosphorylation of pp60$^{v\text{-}src}$ depends on the concentration of ATP present in the reaction. Molar amounts of phosphate were capable of being incorporated into the pp60$^{v\text{-}src}$ molecule when autophosphorylation reactions were conducted at 100 μM ATP (Table 1). This is in contrast to a sixfold-lower extent of phosphorylation of pp60$^{v\text{-}src}$ when reactions were performed under the traditional conditions of very low ATP concentrations. This dependency on ATP concentration for the phosphorylation of pp60$^{v\text{-}src}$ may be correlated with the change in the kinetics of casein phosphorylation that was observed for the A enzyme (Fig. 6C). The change in the kinetic profile of the A enzyme with ATP concentration suggests some type of control of enzyme activity by an ATP/Mg^{2+}-dependent mechanism. During the incubation with ATP/Mg^{2+}, pp60$^{v\text{-}src}$ becomes phosphorylated at tyrosine residues. We have characterized in detail the phosphorylation of pp60$^{v\text{-}src}$ after incubation with concentrations of ATP above and below the K_m of the enzyme for ATP and have found that

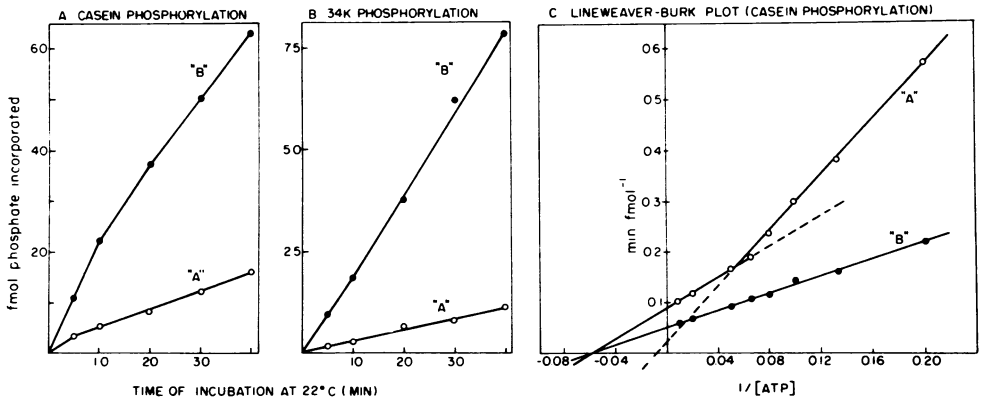

FIG. 6. Reaction kinetics of the phosphorylation of casein and 34K by the pp60$^{v\text{-}src}$ A and B enzymes. (A and B) p60$^{v\text{-}src}$ A enzyme and B enzyme (equal amounts of p60 polypeptide) were incubated in standard analytic protein kinase reaction mixtures containing either casein (A.) or 34K protein preparation (B.). At various times, equal portions were removed and processed as described in the legend to Fig. 2. The numerical values of the phosphorylated protein bands (casein or 34K) are presented. (C) Lineweaver-Burk plot of the phosphorylation of casein by the A and B forms of pp60$^{v\text{-}src}$. The pp60$^{v\text{-}src}$ A and B enzyme preparations were incubated in kinase reaction mixtures containing 1 mg of casein per ml and various concentrations of [γ-^{32}P]ATP (5 to 100 μM; 30 Ci/mmol). At each concentration of ATP, equal portions were removed to SDS-sample buffer after 2, 5, and 10 min of reaction at 22°C. The phosphorylation of casein was quantified by scintillation counting of the phosphorylated protein bands resolved by SDS-polyacrylamide gel electrophoresis. A plot of the phosphorylation at the three time points for each ATP concentration was used to estimate initial reaction velocities (V_0) by extrapolation to time zero. The inverse of V_0 was then plotted against the inverse of the ATP concentrations.

at the higher ATP concentrations a portion of the pp60$^{v\text{-}src}$ molecules were converted to a form that migrated more slowly in SDS-polyacrylamide gels (7). This new form of pp60$^{v\text{-}src}$ was found to be extensively phosphorylated at the site of several new tyrosine residues located on both the amino- and carboxy-terminal regions of the protein, in addition to the major carboxy-terminal tyrosine residue described previously (4, 28). The vast majority of the new phosphorylation, however, occurred on the amino-terminal portion of the molecule. Only when autophosphorylation was conducted at high ATP concentrations were the new sites phosphorylated. Perhaps this phosphorylation, when sufficiently extensive, exerts an allosteric effect on pp60$^{v\text{-}src}$, resulting in a net increase in the protein kinase activity of the enzyme as described in this report.

Snyder et al. (29) recently constructed mutants of RSV in which the tyrosine residue at position 416 in pp60$^{v\text{-}src}$ was replaced by phenylalanine. This is the major phosphorylated tyrosine residue found in pp60$^{v\text{-}src}$ in vivo and is located in the carboxy-terminal region of the polypeptide. This residue is also the major site labeled in vitro when autophosphorylation is carried out at low ATP concentrations (28). The mutant virus was able to transform mouse cells and produce a pp60$^{v\text{-}src}$ molecule capable of phosphorylating protein on tyrosine residues. The authors concluded that phosphorylation of pp60$^{v\text{-}src}$ at tyrosine 416 is not required for pp60$^{v\text{-}src}$ kinase activity or cell transformation (29). It is interesting that tyrosine-specific phosphorylation of the mutant pp60$^{v\text{-}src}$ polypeptide was still detected, indicating that pp60$^{v\text{-}src}$ may be phosphorylated at tyrosine sites other than residue 416. Our correlation of increased specific protein kinase activity, described here, with the appearance of extensive amino-terminal tyrosine phosphorylation on pp60$^{v\text{-}src}$ (7) suggests that phosphorylation (and, by inference, dephosphorylation) of amino-terminal tyrosine residues in pp60$^{v\text{-}src}$ may regulate the function of this transforming protein.

The increase in pp60$^{v\text{-}src}$ specific kinase activity reported here probably represents an underestimate, since our preparations of immunoaffinity-purified pp60$^{v\text{-}src}$ should consist of molecules in various states of phosphorylation. Our incubation with ATP/Mg^{2+} allows us only to compare an extensively phosphorylated preparation of pp60$^{v\text{-}src}$ with a partially phosphorylated enzyme. The phosphotyrosine content of pp60$^{v\text{-}src}$ as isolated from transformed chick cells has been estimated at 0.2 to 0.3 mol/mol of pp60$^{v\text{-}src}$ protein (27). The use of a tyrosine-specific phosphatase should allow us to study the enzymatic activity of totally unphosphorylated pp60$^{v\text{-}src}$.

Even though pp60$^{v\text{-}src}$ was incubated with a large amount of ATP (100 to 500 μM) over a long period of time (60 to 90 min), the repurified pp60$^{v\text{-}src}$ was still able to incorporate label from

[γ-^{32}P]ATP (Fig. 3). Furthermore, ^{32}P-labeled pp60$^{v\text{-}src}$ chromatographed identically to the unphosphorylated enzyme on hexylamine-agarose (unpublished data). Therefore, several alternative explanations for the increase in pp60$^{v\text{-}src}$ specific kinase activity after incubation with ATP/Mg^{2+} should be considered.

One possibility is that the observed effects are not due to enzyme activation at all, but rather that incubation with ATP/Mg^{2+} (with accompanying phosphorylation) may serve to stabilize the enzyme activity. pp60$^{v\text{-}src}$ that has not reacted with ATP/Mg^{2+} may more easily lose its enzymatic activity during subsequent manipulations. In this case, the residual kinase activity present in A enzyme preparations may represent that fraction of pp60$^{v\text{-}src}$ present in the original starting enzyme preparation that has already been so modified. However, we have observed no loss of kinase activity when either starting enzyme, A enzyme or B enzyme preparations have been incubated at 22°C for up to 2 h before the addition of [γ-^{32}P]ATP. Furthermore, this explanation does not explain the continued ability of B enzyme preparations to incorporate radiolabel from [γ-^{32}P]ATP. This latter observation might be due to the presence of a contaminating phosphotyrosine-specific phosphatase in our enzyme preparations. However, our attempts to detect such an activity indicate that if a phosphatase is present, it exists at levels below our detection.

Alternatively, preincubation of the enzyme preparation with ATP/Mg^{2+} may in fact result in an activation of the kinase due to phosphorylation of pp60$^{v\text{-}src}$. However, within the B enzyme preparations, although the vast majority of pp60$^{v\text{-}src}$ molecules have been phosphorylated (and therefore activated), there still exists a small portion of unphosphorylated pp60$^{v\text{-}src}$. These molecules may then serve to incorporate radiolabel from [γ^{-32}P]ATP. Furthermore, since the B enzyme would be much more active than A, the extent of phosphorylation of pp60$^{v\text{-}src}$ in these preparations might appear to be similar, as suggested in Fig. 3.

An alternative explanation for the apparent activation of pp60$^{v\text{-}src}$ by incubation with ATP/Mg^{2+} proposes that such preincubation results in a yet to be identified secondary protein modification of pp60$^{v\text{-}src}$ that does not involve phosphorylation. This modification might be a result of pp60$^{v\text{-}src}$ activity itself, or it may involve another protein (enzyme) present in our partially purified pp60$^{v\text{-}src}$ preparations. ATP/Mg^{2+} may serve as an activator (or substrate) for a second enzyme activity (by phosphorylation or any other ATP/Mg^{2+}-dependent modification), which in turn acts in some stimulatory way on pp60$^{v\text{-}src}$. Conversely, ATP/Mg^{2+} may serve to somehow inactivate or dissociate an inhibitor of pp60$^{v\text{-}src}$ kinase activity.

These and other possible explanations for the results presented here are now under investigation.

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